Award Number: W81XWH-141-0154

TITLE: "A Novel Therapeutic Modality for Advanced-Stage Prostate Cancer Treatment"

PRINCIPAL INVESTIGATOR: Subhash C. Chauhan

CONTRACTING ORGANIZATION: University of Tennessee
                                                    Memphis, TN 38103

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                         Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                          Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE: October 2015	2. REPORT TYPE: Annual Report	3. DATES COVERED: 22 Sep 2014 - 21 Sep 2015

4. TITLE:

"A Novel Therapeutic Modality for Advanced-Stage Prostate Cancer Treatment"

5a. CONTRACT NUMBER:

5b. GRANT NUMBER:
W81XWH-14-1-0154

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S):

Subhash C. Chauhan

E-Mail:schauha1@uthsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES):
University of Tennessee Health Science Center (UTHSC),
62S Dunlap Street Rm 300
Memphis, TN, 38103-4903

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT:
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES: None

14. ABSTRACT: There is an urgent need to develop effective therapies for the treatment of advanced stage prostate cancer (PrCa) due to their limited or no response to androgen ablation therapy. In this proposal, we intend to develop a novel therapeutic agent Ormeloxifene (ORM) for the treatment of advanced stage metastatic PrCa. Our results illustrated that ORM treatment effectively inhibited invasion and motility of PrCa cells. Further, we observed that ORM treatment induced the expression of tumor suppressor PKD1 (a modulator of nuclear β-catenin signaling) in PrCa cells. Interestingly, ORM treatment inhibited expression of oncogenic isoform of PKD (PKD3) in PrCa cells. We have also observed that ORM mediated overexpression/activation of PKD1 effectively inhibits metastasis associated protein 1 (MTA1) in PrCa cells. MTA1 has been reported to be very tightly associated with cancer metastasis in various cancer types including PrCa. To further investigate association of ORM with MTA1 suppression, we performed molecular docking studies with MTA1 which illustrated potential binding sites of ORM on MTA1 protein. Considering effective therapeutic index of ORM, we are also making more potent analogues of ORM. These findings suggest that ORM could be a potential therapeutic molecule to inhibit growth of advanced stage PrCa and its metastasis.

15. SUBJECT TERMS:
Prostate Cancer (PrCa), Metastasis, Ormeloxifene (ORM), Wnt/β-catenin signaling, Androgen Receptor (AR), Estrogen Receptor (ER)

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	UU Unclassified	8	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

**INTRODUCTION**

1. BACKGROUND

Prostate cancer (PrCa) is the second leading cause of cancer death in American men. There is an increasing need to develop effective therapies for advanced stage PrCa due to their limited or no response to androgen ablation therapy [1]. Chemotherapy is an alternative approach for the treatment of advanced stage PrCa. However, the available chemotherapeutic agents used to treat PrCa are non-selective and provide only limited response rate [2]. Thus, novel treatment modalities are needed to treat advanced stage PrCa. In addition, precise understanding of molecular pathogenesis of disease is required to develop novel chemotherapeutic modalities for the treatment of advanced stage PrCa. The androgen receptor (AR) is required for PrCa growth at all stages, including androgen-independent tumors in the presence of very low levels of androgens [3]. Recent studies are suggested that in addition to androgens/AR, estrogens/estrogen receptors (ER) may also play crucial role in the development and progression of PrCa. It is shown that the co-administration of both testosterone and E2 is required for the initiation of PrCa. Additionally, the interaction between β-catenin and the AR and ER suggests a possible mechanism of cross talk between Wnt and androgen/estrogen signaling pathways [4]. Several lines of evidence have shown that β-catenin can act as an AR/ER co-activator and enhance their transcriptional activity [4]. Thus targeting β-catenin-AR/ER signaling pathway by novel chemotherapeutics may have strong clinical implications in developing strategies for PrCa treatment [5]. In recent studies, a novel tri-phenyl ethylene molecule, ormeloxifene, has shown potent anti-cancer activity, including in PrCa cells. ***The central hypothesis of our proposal is that ormeloxifene inhibits growth of advanced stage PrCa cells by modulating β-catenin-AR/ER signaling pathway. Further, it induces cell death via inducing PARP and/or caspase mediated apoptotic pathways.*** The information gathered from this study will provide insight for developing a novel therapeutic modality for advanced stage PrCa. ***To test this hypothesis, the following specific aims were proposed:***

SPECIFIC AIMS:
AIM 1: To examine the effect of ormeloxifene on β-catenin-AR/ER signaling pathways.

AIM 2: Determine the apoptotic pathways activated by ormeloxifene to induce cell death in PrCa cells.

Aim 3: To evaluate the therapeutic efficacy of ormeloxifene for PrCa treatment in mouse model systems.

Ormeloxifene (ORM): Utilization of clinically approved drugs for other indications as anti-cancer agents (repurposing a drug) appears to be an interesting approach because of their established safety profile in human. The ongoing scenario attracts and welcomes the repositioning and budging of existing established drugs which could complement de novo drug development. Ormeloxifene (**Fig. 1**) is a non-hormonal, non-steroidal synthetic molecule for human use as an oral contraceptive [6, 7]. Recently, its anti-cancer activity has been reported against advanced breast cancer [8] and head and neck squamous cell carcinoma (HNSCC) [9]. Additionally, our recent studies show a potent anti-cancer

Figure 1: Chemical structure of ormeloxifene (MW 490.50).

activity of ormeloxifene (ORM) in various cancer cell lines including AR sensitive and AR refractory metastatic PrCa cells. ***Moreover, ORM is reported to have an excellent therapeutic index and is safe for chronic administration*** [10]. Therefore, we believe that ORM has a great repurposing potential for PrCa chemoprevention/treatment. Successful examples of drugs repurposing are anti-diabetic drug metformin and the birth control hormone medroxyprogesterone acetate. In this study we proposed to investigate effects of ORM on β-catenin-AR/ER signaling pathways (**AIM 1**), apoptosis (**AIM 2**) and evaluate its anticancer potential in clinically relevant PrCa cell lines and animal models (**AIM 3**).
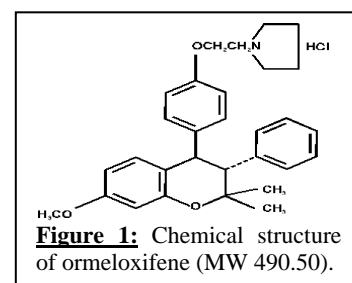
During the 2014-15 funding cycle we made considerable progress on our project. First, we hired the post-doc fellow/Scientist for this project. A selection criterion was based on applicant's track record of publications and

expertise in pre-clinical mouse models of PrCa. We standardized and performed some experiments under specific aims # 1. We performed functional assays to determine the effects of ORM on cell invasion, and cell migration, and colony formation using androgen-independent C4-2 PrCa cell lines. Additionally, we performed some docking studies of ORM with some potential molecular targets of PrCa. We observed that ORM has some potential binding sites with metastasis associated protein 1 (MTA1). We further investigated the effects of ORM on MTA1 protein levels by Western blot analysis. Our results indicate that ORM treatment of C4-2 cells inhibits the protein levels of MTA1. Our lab investigated that MTA1 protein can be inhibited by via ectopic overexpression of protein kinase D1. To investigate whether ORM can induce the expression of PKD1, we performed Western blot analysis, and qRT-PCR to determine the protein levels and mRNA expression of PKD1 in control and ORM treated C4-2 cells. Summary of work done during first year is summarized below.
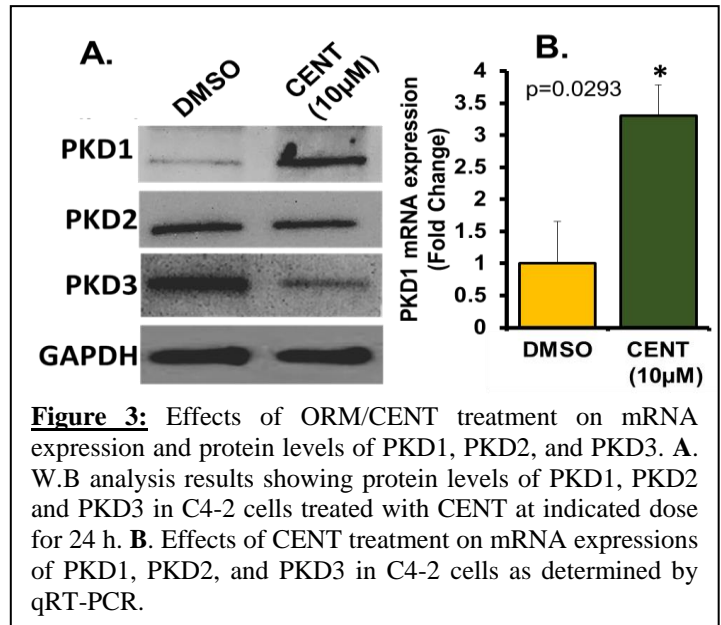
Task 1: To examine the effect of ormeloxifene on β-catenin-AR/ER signaling pathways

1. ORM inhibits invasion and motality of C4-2 cells: To investigate the functional impact of ORM on PrCa cells, we first performed *in vitro* cell invasion assay using chemo-invasion assay kit (BD Biosciences, CA). In this experiment, approximately 70% confluent C4-2 cells were treated with ORM (10 µM) for 24 hours. Equal numbers of viable cells from each group were taken for the assay. All the procedures were followed according to the manufacturer instructions. Our results indicate that ORM significantly inhibited invasion of C4-2 cells **(Fig. 2A).** ORM treatment also inhibited motality of C4-2 cells as determined by scratch wound assay **(Fig.2B).** .
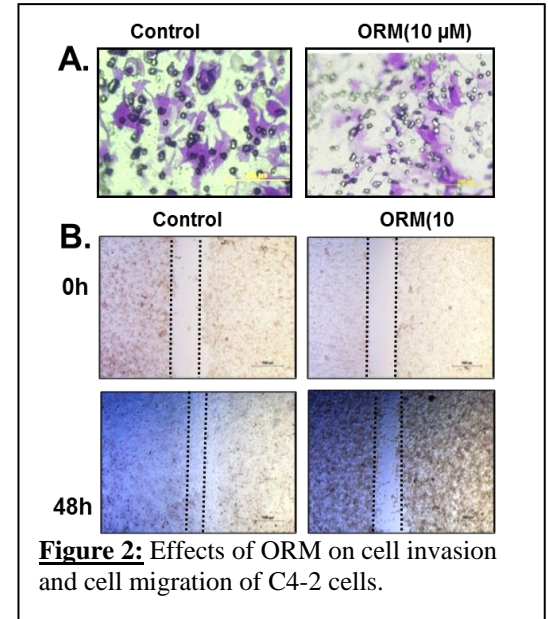

Figure 2: Effects of ORM on cell invasion and cell migration of C4-2 cells.

2. ORM activates tumor suppressive PKD1 and inhibits oncogenic PKD3 in C4-2 cells: Our previous study has shown that activation of PKD1 inhibits Wnt/β-catenin signaling pathways in PrCa cells [11]. These results suggest that PKD1 can be activated or overexpressed pharmacologically, thus PKD1 inducer could be use in clinic for therapeutic purpose. Bryostatin-1 use in clinic has some limitations because of its toxicity and low bioavailability [12]. Thus, we were interested to explore whether inducing expression of PKD1 by ORM treatment can inhibit Wnt/ β-catenin signaling pathways in PrCa cells. In this experiment, we treated approximately 70% confluent C4-2 cells with ORM (10 µM) for 24 hours. Cell lysates and RNA were prepared for Western blot and qRT-PCR analyses to determine the effects of ORM protein and mRNA expression of PKD1. Our


Figure 3: Effects of ORM/CENT treatment on mRNA expression and protein levels of PKD1, PKD2, and PKD3. **A**. W.B analysis results showing protein levels of PKD1, PKD2 and PKD3 in C4-2 cells treated with CENT at indicated dose for 24 h. **B**. Effects of CENT treatment on mRNA expressions of PKD1, PKD2, and PKD3 in C4-2 cells as determined by qRT-PCR.

results indicate that ORM (10 µM) treatment induces both mRNA and protein levels of PKD1 in C4-2 cells **(Fig. 3A, B).** To determine whether this effect of ORM in PrCa cells is specific to PKD1, we investigated the effects of ORM on other isoforms of PKD (PKD1 and PKD2). Interestingly, we observed that ORM treatment inhibits expression of oncogenic PKD3 **(Fig. 3A, B).** However, no effect was observed on the mRNA expression and protein levels of PKD2 **(Fig. 3A, B).**

3. ORM interacts with and inhibits protein levels of Metastasis Associated Protein 1 (MTA1): We performed docking study to find whether CENT interacts with MTA1 protein. The Grid Docking was done with 4BKX. We found that ORM has four potential binding sites on MTA1 protein (**Fig 4A**). The one with the best score is located at 270 SER (ELM2 dimerization) owing to its interactions with MTA1 and relative deep binding site inside the pocket (**Fig. 4B**). The one with two H bond interactions with MTA1 is located at 170 ASP and 181 LEU (ELM2 motif). These results suggest that ORM may directly interact with MTA1. We further determine the effects of ORM on MTA1 protein levels. As shown in **Fig. 5A,** ORM treatment inhibits protein levels of MTA1 in C4-2 cells. We are doing more docking experiments to find out the potential molecular targets of ORM in PrCa.
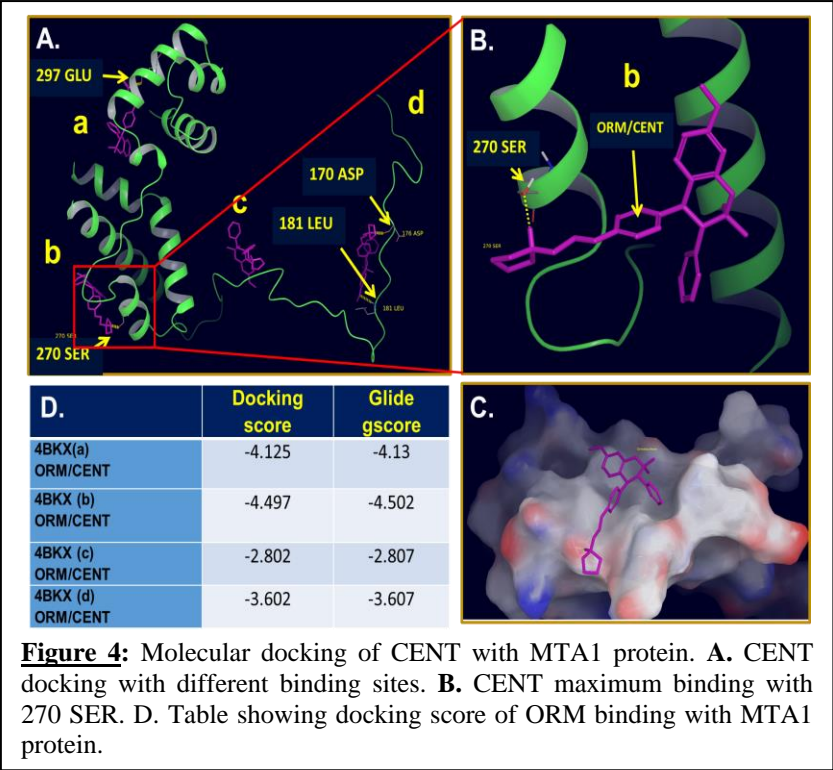
Figure 4: Molecular docking of CENT with MTA1 protein. **A.** CENT docking with different binding sites. **B.** CENT maximum binding with 270 SER. D. Table showing docking score of ORM binding with MTA1 protein.

5. ORM enhances the sensitivity of chemotherapeutic drug Docetaxel (DTX): It has been reported that MTA1 is involved in DTX drug resistance [13] and DTX treatment does not inhibit the expression of MTA1 in PrCa cells (**Fig. 5A).** However, ORM treatment effectively inhibited the expression of MTA1 (**Fig. 5A**). Thus, we hypothesized that ORM treatment may enhance the DTX sensitivity in PrCa cells. To prove our hypothesis, we performed cell proliferation and colony formation assays in C4-2 cells treated alone or in combination with DTX. Results illustrated that ORM treatment potentiates the effects of DTX as determined by MTS (**Fig. 5B**). Further our colony formation results also depicted significant (P<0.05) reduction in colony formation compared with alone ORM and DTX treatment. These results indicate that ORM has potential to induce the DTX effects in PrCa. ORM in combination with Overall, these results suggest that ORM which induces PKD1 expression and inhibit MTA1 can sensitize the DTX resistance in PrCa.
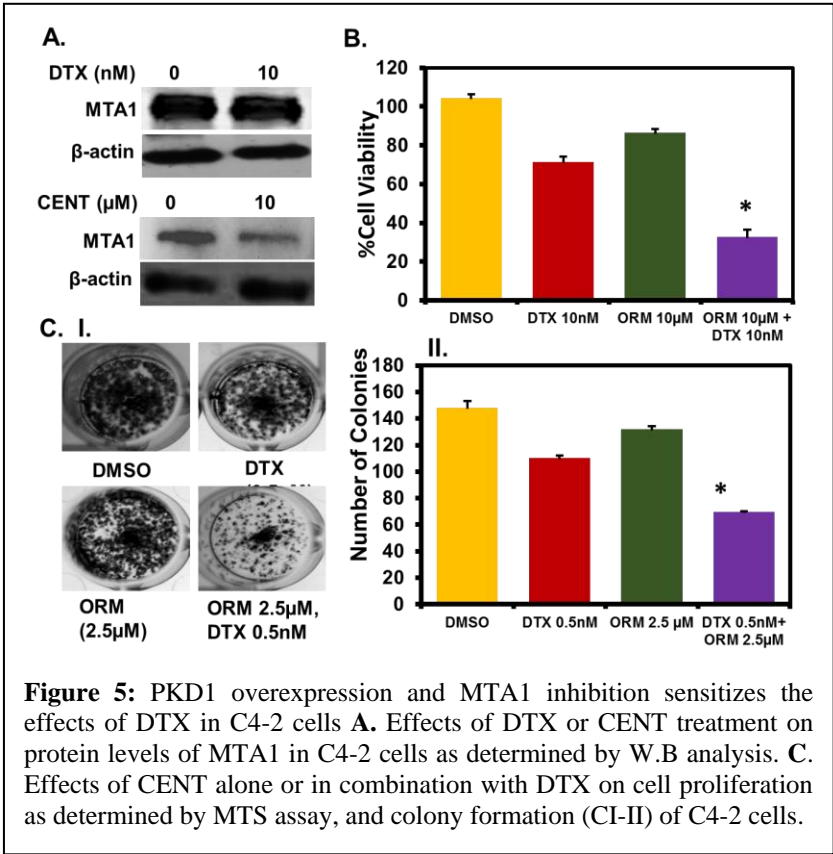
Figure 5: PKD1 overexpression and MTA1 inhibition sensitizes the effects of DTX in C4-2 cells **A.** Effects of DTX or CENT treatment on protein levels of MTA1 in C4-2 cells as determined by W.B analysis. **C.** Effects of CENT alone or in combination with DTX on cell proliferation as determined by MTS assay, and colony formation (CI-II) of C4-2 cells.

Ongoing Experiments for Task 1:

Experiments are continued to determine the effects of ORM treatment on Wnt/β-catenin signaling pathways. For this, we have generated C4-2 stable cell line expressing β-catenin using nucleus targeted red fluorescent

protein (tdTomato, Clontech) fused β-catenin constructs. Experiments are continued to determine the effects of ORM on subcellular localization of β-catenin by confocal laser scanning microscopy. Experiments are also continued to generate more PrCa overexpressing β-catenin stable cell lines using the same technique. Similar experiments will be performed in these cell lines to determine the effects of ORM on subcellular localization of β-catenin by confocal laser scanning microscopy.

Future Experiments for Task 1:

1. We have shown that overexpression of PKD1 inhibits Wnt/β-catenin signaling pathways in PrCa cells. We are expecting that ORM treatment will inhibit Wnt/β-catenin signaling pathways through activation of PKD1. It may be possible that ORM directly interact with β-catenin protein. Therefore, we are also doing docking experiments to find out potential binding sites of ORM on β-catenin protein.

2. Effect of ORM on androgen receptor (AR) and estrogen receptor (ER) signaling pathways. We will determine whether ORM treatment inhibits the transactivation of AR and ER. For this, we plan to determine the effects of ORM treatment on AR and ER luciferase activity. We will also determine whether ORM treatment inhibits AR translocation from cytoplasm to nucleus by confocal microscopy. Further, we will determine the effects of

3. Effects of ORM on tumor microenvironment: We will determine the effects of ORM on EMT markers (E-cadherin, Vimentin, and Slug) in PrCa cells. This will be analyzed by Western blot analysis and qRT-PCR analyses.

Task 2: *Determine the apoptotic pathways activated by ormeloxifene to induce cell death in PrCa cells*

Future Experiments for Task 2: We have standardized apoptotic experiments in our lab. Under Task #2, we will determine whether and how ORM treatment induces apoptosis in PrCa cells. Following experiments will be performed to accomplish this task # 2.

1. Effects of ORM on apoptosis by Tunnel Assay to determine the apoptotic inducing potential of ORM in PrCa cells.
2. Effects of ORM on extrinsic and intrinsic apoptotic pathways proteins to determine which apoptotic pathway are involved in induction of apoptosis by ORM in PrCa cells. For extrinsic pathway analysis, we will analyze TNFR1, Fas, DR-3, DR-4, DR-5 and DR-6 and for intrinsic pathways, we will determine the effects of ORM on Bcl2, Bax, and Cytochrome C.
3. Effect of ORM on caspases 3 and 9 and cleaved PARP and total PARP by Western blot analysis.

Task 3: **To evaluate the therapeutic efficacy of ORM for PrCa treatment in mouse model systems**

Experiments done for Task 3:

Generation of TRAMP mouse model: We have successfully generated and characterized TRAMP mouse model in our lab. As shown in Fig 6A TRAMP mouse showing prostate tumor at 22 weeks of age. We investigated the expression of PKD1 and MTA1 in prostate tissues of 8 and 24 weeks old TRAMP mice and their littermates. We observed loss of PKD1 expression in TRAMP mice prostate compared to the wild type as determined by Western blot and q-RTPCR analysis **(Fig. 6 A, B).** Further, we also observed that MTA1 which is regulated by PKD1 was overexpressed in the prostate of TRAMP mice compared to wild type littermate. These results suggest that loss of PKD1 and overexpression of MTA1 proteins correlates with the progression of prostate tumor in TRAMP mouse model.
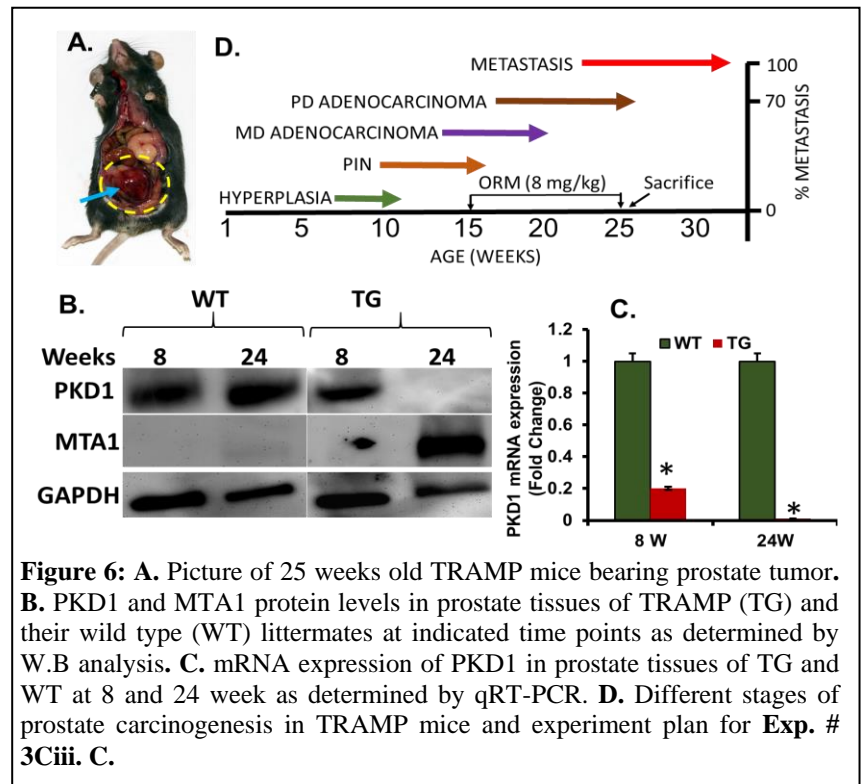
Future Experiments for Task 3:

We will perform the chemopreventive potential of ORM in TRAMP mice. Because ORM treatment induces expression of PKD1 and inhibits MTA1 proteins in PrCa cells. Therefore, we expect that ORM treatment will inhibit the prostate tumor growth and metastasis in TRAMP mice. We will also determine the therapeutic efficacy of ORM in ectopic xenograft mouse model of PrCa.

Summary of Research Accomplishment during 1st year of the project.

1. Determined the effects of ORM in cell invasion and motility of PrCa cells.
2. Investigated the effects of ORM on tumor suppressor protein (PKD1) and oncogenic protein (MTA1).
3. Performed functional assays (Cell proliferation and colony formation) to investigate the effects of ORM on DTX sensitivity.
4. Performed in-silico analysis to determine the potential binding sites of ORM on MTA1 protein.
5. Generated and characterized TRAMP mouse model.

Figure 6: A. Picture of 25 weeks old TRAMP mice bearing prostate tumor. **B.** PKD1 and MTA1 protein levels in prostate tissues of TRAMP (TG) and their wild type (WT) littermates at indicated time points as determined by W.B analysis. **C.** mRNA expression of PKD1 in prostate tissues of TG and WT at 8 and 24 week as determined by qRT-PCR. **D.** Different stages of prostate carcinogenesis in TRAMP mice and experiment plan for **Exp. # 3Ciii. C.**

References:

1. Damber, J.E. and G. Aus, *Prostate cancer.* Lancet, 2008. **371**(9625): p. 1710-21.
2. Sahoo, S.K., W. Ma, and V. Labhasetwar, *Efficacy of transferrin-conjugated paclitaxel-loaded nanoparticles in a murine model of prostate cancer.* Int J Cancer, 2004. **112**(2): p. 335-40.
3. Kaarbo, M., T.I. Klokk, and F. Saatcioglu, *Androgen signaling and its interactions with other signaling pathways in prostate cancer.* Bioessays, 2007. **29**(12): p. 1227-38.
4. Verras, M. and Z. Sun, *Roles and regulation of Wnt signaling and beta-catenin in prostate cancer.* Cancer Lett, 2006. **237**(1): p. 22-32.
5. Sarkar, F.H., et al., *Novel targets for prostate cancer chemoprevention.* Endocr Relat Cancer, 2010. **17**(3): p. R195-212.
6. Kamboj, V.P., et al., *Biological profile of Centchroman--a new post-coital contraceptive.* Indian J Exp Biol, 1977. **15**(12): p. 1144-50.
7. Misra, N.C., et al., *Centchroman--a non-steroidal anti-cancer agent for advanced breast cancer: phase-II study.* Int J Cancer, 1989. **43**(5): p. 781-3.
8. Nigam, M., et al., *Centchroman induces G0/G1 arrest and caspase-dependent apoptosis involving mitochondrial membrane depolarization in MCF-7 and MDA MB-231 human breast cancer cells.* Life Sci, 2008. **82**(11-12): p. 577-90.
9. Srivastava, V.K., et al., *Centchroman inhibits proliferation of head and neck cancer cells through the modulation of PI3K/mTOR pathway.* Biochem Biophys Res Commun, 2011. **404**(1): p. 40-5.
10. Singh, M.M., *Centchroman, a selective estrogen receptor modulator, as a contraceptive and for the management of hormone-related clinical disorders.* Med Res Rev, 2001. **21**(4): p. 302-47.
11. Jaggi, M., et al., *Bryostatin 1 modulates beta-catenin subcellular localization and transcription activity through protein kinase D1 activation.* Mol Cancer Ther, 2008. **7**(9): p. 2703-12.
12. Marshall, J.L., et al., *Phase I study of prolonged infusion Bryostatin-1 in patients with advanced malignancies.* Cancer Biol Ther, 2002. **1**(4): p. 409-16.
13. Yu, L., et al., *Repression of NR4A1 by a chromatin modifier promotes docetaxel resistance in PC-3 human prostate cancer cells.* FEBS Lett, 2013. **587**(16): p. 2542-51.